

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
11 April 2002 (11.04.2002)

PCT

(10) International Publication Number
WO 02/28480 A2

- (51) International Patent Classification⁷: **A61P 35/00**,
A61K 39/395 // C07K 16/28
- (21) International Application Number: **PCT/US01/30961**
- (22) International Filing Date: **2 October 2001 (02.10.2001)**
- (25) Filing Language: **English**
- (26) Publication Language: **English**
- (30) Priority Data:
60/237,556 **2 October 2000 (02.10.2000)** **US**
- (71) Applicant (for all designated States except US): **CHIRON CORPORATION** [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US).
- (72) Inventors; and
- (75) Inventors/Applicants (for US only): **CHU, Keting** [US/US]; Chiron Corporation, P.O. Box 8097, Emeryville, CA 94622-8097 (US). **MASUOKA, Lorianne** [US/US]; Chiron Corporation, P.O. Box 8097, Emeryville, CA 94622-8097 (US).
- (74) Agents: **ALEXANDER, Lisa, E.**; Chiron Corporation, Intellectual Property, P.O. Box 8097, Emeryville, CA 94662-8097 et al. (US).
- (81) Designated States (*national*): AE, AG, AL, AM, AT, AT (utility model), AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, CZ (utility model), DE, DE (utility model), DK, DK (utility model), DM, DZ, EC, EE, EE (utility model), ES, FI, FI (utility model), GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PH, PT, RO, RU, SD, SE, SG, SI, SK, SK (utility model), SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA.
- (84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).
- Published:**
— *without international search report and to be republished upon receipt of that report*
- For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.*

(54) Title: METHODS OF THERAPY FOR B-CELL MALIGNANCIES

(57) Abstract: Methods of therapy for B-cell malignancies are provided. The methods comprise administering a therapeutically effective amount of an antagonist anti-CD40 antibody or antigen-binding fragment thereof to a patient in need thereof. The antagonist anti-CD40 antibody or antigen-binding fragment thereof is free of significant agonist activity when the antibody binds a CD40 antigen on a normal human B cell, exhibits antagonist activity when the antibody binds a CD40 antigen on a malignant human B cell, and can exhibit antagonist activity when the antibody binds a CD40 antigen on a normal human B cell. Antagonist activity of the anti-CD40 antibody or antigen-binding fragment thereof beneficially inhibits proliferation and/or differentiation of malignant human B cells.

WO 02/28480 A2

METHODS OF THERAPY FOR B-CELL MALIGNANCIES

FIELD OF THE INVENTION

The present invention is directed to methods of therapy for diseases characterized by malignant B cells and tumors of B-cell origin using antagonist anti-CD40 antibodies or antigen-binding fragments thereof.

5 BACKGROUND OF THE INVENTION

B cells play an important role during the normal *in vivo* immune response. A foreign antigen will bind to surface immunoglobulins on specific B cells, triggering a chain of events including endocytosis, processing, presentation of processed peptides on MHC-class II molecules, and up-regulation of the B7 antigen on the B-cell surface. 10 A specific T-cell then binds to the B cell via T-cell receptor (TCR) recognition of the processed antigen presented on the MHC-class II molecule. Stimulation through the TCR activates the T cell and initiates T-cell cytokine production. A second signal that further activates the T cell is an interaction between the CD28 antigen on T cells and the B7 antigen on B cells. When the above-mentioned signals are received, the 15 CD40 ligand, which is not expressed on resting human T cells, is up-regulated on the T-cell surface. Binding of the CD40 ligand to the CD40 antigen on the B-cell surface stimulates the B cell, causing the B cell to mature into a plasma cell secreting high levels of soluble immunoglobulin.

CD40 is a cell-surface antigen present on the surface of both normal and 20 neoplastic human B cells, dendritic cells, monocytic and epithelial cells, some epithelial carcinomas, and on antigen presenting cells (APCs). CD40 expression on APCs plays an important co-stimulatory role in the activation of both T helper and cytotoxic T lymphocytes. CD40 receptors are also found on eosinophils, synovial membranes in rheumatoid arthritis, activated platelets, inflamed vascular endothelial 25 cells, dermal fibroblasts, and other non-lymphoid cell types. The CD40 receptor is expressed on activated T cells, activated platelets, and inflamed vascular smooth

muscle cells. CD40 is also expressed at low levels on vascular endothelial cells and is up-regulated in areas of local inflammation.

Human CD40 is a peptide of 277 amino acids having a predicted molecular weight of 30,600, with a 19 amino acid secretory signal peptide comprising
5 predominantly hydrophobic amino acids. The CD40 receptor exists in a highly modified glycoprotein state on the cell surface and migrates in sodium dodecyl sulfate (SDS)-polyacrylamide gels as an approximately 50 kDa polypeptide.

The CD40 antigen is known to be related to the human nerve growth factor (NGF) receptor, tumor necrosis factor- α (TNF- α) receptor, and Fas, suggesting that
10 CD40 is a receptor for a ligand with important functions in B-cell activation. During B-cell differentiation, the molecule is first expressed on pre-B cells and then disappears from the cell surface when the B cell becomes a plasma cell. The CD40 cell-surface antigen plays an important role in B-cell proliferation and differentiation.

Binding of its ligand (termed CD40L or CD154) to the CD40 receptor
15 stimulates B-cell proliferation and differentiation, antibody production, isotype switching, and B-cell memory generation. The human and murine CD40L (CD40 receptor) genes have been cloned (Spriggs *et al.* (1992) *J. Exp. Med.* 176:1543; Armitage *et al.* (1992) *Nature* 357:80; and U.S. Patent No. 6,264,951). Engagement of CD40 receptors by the CD40 ligand on APCs, such as macrophages and dendritic
20 cells, up-regulates cell-surface expression of MHC Class II and CD80/86, and induces the secretion of pro-inflammatory cytokines such as IL-8, IL-12, and TNF, all of which increase the potency of antigen presentation to T cells.

All B cells express common cell surface markers, including CD40. Transformed cells from patients with low- and high-grade B-cell lymphomas, B-cell
25 acute lymphoblastic leukemia, multiple myeloma, chronic lymphocytic leukemia, and Hodgkin's disease express CD40. CD40 expression is also detected in two-thirds of acute myeloblastic leukemia cases and 50% of AIDS-related lymphomas. Further, malignant B cells from several tumors of B-cell lineage express a high degree of CD40 and appear to depend on CD40 signaling for survival and proliferation.

30 Additionally, immunoblastic B-cell lymphomas frequently arise in immunocompromised individuals such as allograft recipients and others receiving long-term immunosuppressive therapy, AIDS patients, and patients with primary immunodeficiency syndromes such as X-linked lymphoproliferative syndrome or

Wiscott-Aldrich syndrome (Thomas *et al.* (1991) *Adv. Cancer Res.* 57:329; Straus *et al.* (1993) *Ann. Intern. Med.* 118:45). These tumors appear to arise as a result of impaired T-cell control of latent Epstein-Barr virus (EBV) infection. Similar lymphomas of human origin can be induced in mice with severe combined
5 immunodeficiency syndrome (SCID) by inoculation of peripheral blood lymphocytes (PBL) from healthy, EBV-positive individuals (Mosier *et al.* (1988) *Nature* 335:256; Rowe *et al.* (1991) *J. Exp. Med.* 173:147).

The pathogenesis of low-grade B-lineage malignancies, including non-Hodgkin's lymphoma and chronic lymphocytic leukemia, is strongly affected by the
10 imbalance of the growth/survival signal by CD40 and a crippled death signal by Fas. Studies in low-grade non-Hodgkin's lymphoma suggest that the disease is the result of an accumulation of lymphomatous cells due to reduction in Fas-mediated apoptosis and an increase in the survival signal through CD40. CD40 provides a survival signal for lymphoma cells from non-Hodgkin's B-lymphoma patients and stimulates their
15 growth *in vitro* (Romano *et al.* (2000) *Leuk. Lymphoma* 36:255-262; Furman *et al.* (2000) *J. Immunol.* 164:2200-2206; Kitada *et al.* (1999) *Br. J. Haematol.* 106:995-1004; Romano *et al.* (1998) *Blood* 92:990-995; Jacob *et al.* (1998) *Leuk. Res.* 22:379-382; Wang *et al.* (1997) *Br. J. Haematol.* 97:409-417; Planken *et al.* (1996) *Leukemia* 10:488-493; and Greiner *et al.* (1997) *Am J. Pathol.* 150:1583-1593).

Approximately 85% of non-Hodgkin's lymphomas, a diverse group of malignancies, are of B-cell origin. The non-Hodgkin's lymphomas originate from components of the spleen, thymus, and lymph nodes. In the *Working Formulation* classification scheme, these lymphomas been divided into low-, intermediate-, and high-grade categories by virtue of their natural histories (see "The Non-Hodgkin's
25 Lymphoma Pathologic Classification Project," *Cancer* 49(1982):2112-2135). The low-grade or favorable lymphomas are indolent, with a median survival of 5 to 10 years (Horning and Rosenberg (1984) *N. Engl. J. Med.* 311:1471-1475). Although chemotherapy can induce remissions in the majority of indolent lymphomas, cures are rare, and most patients eventually relapse, requiring further therapy. The
30 intermediate- and high-grade lymphomas are more aggressive tumors, but they have a greater chance for cure with chemotherapy. However, significant numbers of these patients will still relapse and require further treatment to induce remissions.

Furthermore, patients undergoing chemotherapy can experience toxicity effects. Therefore, there is a need for new therapies for treating diseases of malignant B cells.

SUMMARY OF THE INVENTION

5 Methods for treating a patient with a disease comprising malignant B cells, including lymphomas such as non-Hodgkin's lymphomas (high-grade lymphomas, intermediate-grade lymphomas, and low-grade lymphomas), Hodgkin's disease, acute lymphoblastic leukemias, myelomas, chronic lymphocytic leukemias, and myeloblastic leukemias are provided. The method comprises treating the patient with
10 anti-CD40 antibodies or antigen-binding fragments thereof that are free of significant agonist activity when bound to a CD40 antigen on a normal human B cells and that exhibit antagonist activity when bound to a CD40 antigen on a malignant human B cell. Monoclonal antibodies and antigen-binding fragments thereof that are suitable for use in the methods of the invention exhibit the following characteristics: 1) are
15 capable of specifically binding to a human CD40 antigen expressed on the surface of a human cell; 2) are free of significant agonist activity when bound to a CD40 antigen on a normal human B cell; and, 3) exhibit antagonist activity when bound to a CD40 antigen on a malignant human B cell. In some embodiments, the anti-CD40 antibody or fragment thereof also exhibits antagonist activity when bound to CD40 antigen on
20 normal human B cells. The monoclonal antibodies have a strong affinity for CD40 and are characterized by a dissociation constant (K_d) of at least 10^{-5} M, preferably at least about 10^{-8} M to about 10^{-20} M, more preferably at least about 5×10^{-9} to about 10^{-16} M. Suitable monoclonal antibodies have human constant regions; preferably they also have wholly or partially humanized framework regions; and most preferably
25 are fully human antibodies or antigen-binding fragments thereof.

 In one embodiment of the invention, the therapy comprises administering to a patient a therapeutically effective dose of a pharmaceutical composition comprising suitable anti-CD40 antibodies or antigen-binding fragments thereof. A therapeutically effective dose of the anti-CD40 antibody or fragment thereof is in the range from
30 about 0.01 mg/kg to about 40 mg/kg, from about 0.01 mg/kg to about 30 mg/kg, from about 0.1 mg/kg to about 30 mg/kg, from about 1 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 30 mg/kg, from about 3 mg/kg to about 25 mg/kg, from about 3 mg/kg to about 20 mg/kg, from about 5 mg/kg to about 15 mg/kg, or from about 7

mg/kg to about 12 mg/kg. It is recognized that the treatment may comprise administration of a single therapeutically effective dose or administration of multiple therapeutically effective doses of the anti-CD40 antibody or antigen-binding fragment thereof.

- 5 The anti-CD40 antibodies suitable for use in the methods of the invention may be modified. Modifications of the anti-CD40 antibodies include, but are not limited to, immunologically active chimeric anti-CD40 antibodies, humanized anti-CD40 antibodies, and immunologically active murine anti-CD40 antibodies.

10 DETAILED DESCRIPTION OF THE INVENTION

- The present invention is directed to methods for treating human patients with diseases that originate from malignant B cells. The methods involve treatment with an anti-CD40 antibody or antigen-binding fragment thereof, where administration of the antibody or antigen-binding fragment thereof promotes a positive therapeutic
- 15 response within the patient undergoing this method of therapy. Anti-CD40 antibodies suitable for use in the methods of the invention have the following characteristics: 1) they specifically bind a human CD40 antigen expressed on the surface of a human cell; 2) they are free of significant agonist activity when bound to a CD40 antigen on a normal human B cell; and 3) they exhibit antagonist activity when bound to a CD40
- 20 antigen on a malignant human B cell. These anti-CD40 antibodies and antigen-binding fragments thereof are referred to herein as antagonist anti-CD40 antibodies. Such antibodies include, but are not limited to, the fully human monoclonal antibody 15B8 described below and monoclonal antibodies having the binding characteristics of monoclonal antibody 15B8. As discussed in more detail below, these antibodies
- 25 are specific to CD40 receptors. When these antibodies bind CD40 displayed on the surface of normal human B cells, the antibodies are free of significant agonist activity; in some embodiments, their binding to CD40 displayed on the surface of normal human B cells results in inhibition of proliferation and differentiation of these normal human B cells. Thus, the antagonist anti-CD40 antibodies suitable for use in the
- 30 methods of the invention include those monoclonal antibodies that can exhibit antagonist activity toward normal human B cells expressing the cell-surface CD40 antigen. When antagonist anti-CD40 antibodies bind CD40 displayed on the surface

of malignant human B cells, the antibodies exhibit antagonist activity as defined elsewhere herein.

"Treatment" is herein defined as the application or administration of an antagonist anti-CD40 antibody or antigen-binding fragment thereof to a patient, or application or administration of an antagonist anti-CD40 antibody or fragment thereof to an isolated tissue or cell line from a patient, where the patient has a disease, a symptom of a disease, or a predisposition toward a disease, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of the disease, or the predisposition toward the disease. By "treatment" is also intended the application or administration of a pharmaceutical composition comprising the antagonist anti-CD40 antibodies or fragments thereof to a patient, or application or administration of a pharmaceutical composition comprising the anti-CD40 antibodies or fragments thereof to an isolated tissue or cell line from a patient, who has a disease, a symptom of a disease, or a predisposition toward a disease, where the purpose is to cure, heal, alleviate, relieve, alter, remedy, ameliorate, improve, or affect the disease, the symptoms of the disease, or the predisposition toward the disease.

By "anti-tumor activity" is intended a reduction in the rate of malignant B-cell proliferation or accumulation, and hence a decline in growth rate of an existing tumor or in a tumor that arises during therapy, and/or destruction of existing neoplastic (tumor) cells or newly formed neoplastic cells, and hence a decrease in the overall size of a tumor during therapy. Therapy with at least one anti-CD40 antibody (or antigen-binding fragment thereof) causes a physiological response that is beneficial with respect to treatment of disease states comprising malignant B cells in a human.

The monoclonal antibody 15B8 represents a suitable antagonist anti-CD40 antibody for use in the methods of the present invention. This antibody is described in U.S. Provisional Application Serial No. 60/237,556, titled "Human Anti-CD40 Antibodies," filed October 2, 2000, and PCT International Application No. PCT/US01/_____, also titled "Human Anti-CD40 Antibodies," filed October 2, 2001 (Attorney Docket No. PP15798.002), both of which are herein incorporated by reference in their entirety. The 15B8 antibody is a fully human anti-CD40 monoclonal antibody of the IgG₂ isotype produced from the hybridoma cell line 15B8. The cell line was created using splenocytes from an immunized xenotypic

mouse containing a human immunoglobulin locus (Abgenix). The spleen cells were fused with the mouse myeloma SP2/0 cells (Sierra BioSource). The resulting hybridomas were sub-cloned several times to create the stable monoclonal cell line 15B8.

5 The 15B8 cell line was adapted to grow in protein-free medium and used to create a Master Cell Bank. The Master Cell Bank was tested for identity and adventitious and endogenous contaminants. The Master Cell Bank was used to manufacture the desired human IgG₂. The respective 15B8 antibody was purified using chromatography and filtration procedures.

10 The anti-CD40 antibody 15B8 is a polypeptide composed of 1,284 amino acid residues with a predicted molecular weight of 149,755 with two heavy chains and two light chains in a heterodimeric arrangement. Amino acid analysis reveals that the antibody is composed of equimolar amounts of heavy and light chains. The 15B8 monoclonal antibody binds soluble CD40 in ELISA-type assays. When tested *in vitro*
15 for effects on proliferation of B cells from numerous primates, 15B8 acts as an agonistic anti-CD40 antibody in cynomologous, baboon, and rhesus monkeys. In assays with humans, chimpanzees, and marmosets, 15B8 is an antagonist anti-CD40 antibody. The binding affinity of 15B8 to human CD40 is 3.1×10^{-9} M as determined by the Biacore™ assay.

20 Suitable antagonist anti-CD40 antibodies for use in the methods of the present invention exhibit a strong single-site binding affinity for the CD40 cell-surface antigen. The monoclonal antibodies of the invention exhibit a dissociation constant (K_d) for CD40 of at least 10^{-5} M, at least 3×10^{-5} M, preferably at least 10^{-6} M to 10^{-7} M, more preferably at least 10^{-8} M to about 10^{-20} M, yet more preferably at least $5 \times$
25 10^{-9} M to about 10^{-18} M, most preferably at least about 5×10^{-9} M to about 10^{-16} M, such as 10^{-8} M, 5×10^{-9} M, 10^{-9} M, 5×10^{-10} M, 10^{-10} M, 5×10^{-11} M, 10^{-11} M, 5×10^{-12} M, 10^{-12} M, 5×10^{-13} M, 10^{-13} M, 5×10^{-14} M, 10^{-14} M, 5×10^{-15} M, 10^{-15} M, 5×10^{-16} M, or 10^{-16} M, as measured using a standard assay such as Biacore™. Biacore analysis is known in the art and details are provided in the "BIAapplications
30 handbook."

By "CD40 antigen" is intended a glycosylated transmembrane peptide or any fragment thereof (GenBank Accession No. X60592; U.S. Patent Nos. 5,674,492 and 4,708,871; Stamenkovic *et al.* (1989) *EMBO* 8:1403; Clark (1990) *Tissue Antigens*

36:33; Barclay *et al.* (1997) *The Leucocyte Antigen Facts Book* (2d ed.; Academic Press, San Diego)). The CD40 receptor is displayed on the surface of a variety of cell types, as described elsewhere herein. By "displayed on the surface" and "expressed on the surface" is intended that all or a portion of the CD40 antigen is exposed to the exterior of the cell. The displayed or expressed CD40 antigen may be fully or partially glycosylated.

By "agonist activity" is intended that the substance functions as an agonist. An agonist combines with a receptor on a cell and initiates a reaction or activity that is similar to or the same as that initiated by the receptor's natural ligand. An agonist of CD40 induces any or all of, but not limited to, the following responses: B-cell proliferation and differentiation, antibody production, intercellular adhesion, B-cell memory generation, isotype switching, up-regulation of cell-surface expression of MHC Class II and CD80/86, and secretion of pro-inflammatory cytokines such as IL-8, IL-12, and TNF. By "antagonist activity" is intended that the substance functions as an antagonist. An antagonist of CD40 prevents or reduces induction of any of the responses induced by binding of the CD40 receptor to an agonist ligand, particularly CD40L. The antagonist may reduce induction of any one or more of the responses to agonist binding by 5%, 10%, 15%, 20%, 25%, 30%, 35%, preferably 40%, 45%, 50%, 55%, 60%, more preferably 70%, 80%, 85%, and most preferably 90%, 95%, 99%, or 100%. Methods for measuring B-cell responses are known to one of skill in the art and include, but are not limited to, B-cell proliferation assays, Banchereau-Like-B-Cell proliferation assays, T-cell helper assays for antibody production, co-stimulation of B-cell proliferation assays, and assays for up-regulation of B-cell activation markers. Several of these assays are discussed in more detail elsewhere herein.

By "significant" agonist activity is intended an agonist activity of at least 30%, 35%, 40%, 45%, 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, or 100% greater than the agonist activity induced by a neutral substance or negative control as measured in an assay of a B-cell response. A substance "free of significant agonist activity" would exhibit an agonist activity of not more than about 25% greater than the agonist activity induced by a neutral substance or negative control, preferably not more than about 20% greater, 15% greater, 10% greater, 5% greater, 1% greater, 0.5% greater, or even not more than about 0.1% greater than the agonist activity induced by a

neutral substance or negative control as measured in an assay of a B-cell response. The antagonist anti-CD40 antibodies useful in the methods of the present invention are free of significant agonist activity as noted above when bound to a CD40 antigen on a normal human B cell. In one embodiment of the invention, the antagonist anti-
5 CD40 antibody is free of significant agonist activity in one B-cell response. In another embodiment of the invention, the antagonist anti-CD40 antibody is free of significant agonist activity in assays of more than one B-cell response (e.g., proliferation and differentiation, or proliferation, differentiation, and antibody production).

10 As used herein "anti-CD40 antibody" encompasses any antibody that specifically recognizes the CD40 B-cell surface antigen, including polyclonal antibodies, monoclonal antibodies, single-chain antibodies, and fragments thereof such as Fab, F(ab')₂, F_v, and other fragments which retain the antigen binding function of the parent anti-CD40 antibody. Polyclonal sera may be prepared by conventional
15 methods. In general, a solution containing the CD40 antigen is first used to immunize a suitable animal, preferably a mouse, rat, rabbit, or goat. Rabbits or goats are preferred for the preparation of polyclonal sera due to the volume of serum obtainable, and the availability of labeled anti-rabbit and anti-goat antibodies. Polyclonal sera can be prepared in a transgenic animal, preferably a mouse bearing
20 human immunoglobulin loci. In a preferred embodiment, Sf9 cells expressing CD40 are used as the immunogen. Immunization can also be performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally (generally subcutaneously or intramuscularly). A dose of 50-200 µg/injection is
25 typically sufficient. Immunization is generally boosted 2-6 weeks later with one or more injections of the protein in saline, preferably using Freund's incomplete adjuvant. One may alternatively generate antibodies by *in vitro* immunization using methods known in the art, which for the purposes of this invention is considered equivalent to *in vivo* immunization. Polyclonal antisera are obtained by bleeding the
30 immunized animal into a glass or plastic container, incubating the blood at 25°C for one hour, followed by incubating at 4°C for 2-18 hours. The serum is recovered by centrifugation (e.g., 1,000 x g for 10 minutes). About 20-50 ml per bleed may be obtained from rabbits.

Preferably the antibody is monoclonal in nature. By "monoclonal antibody" is intended an antibody obtained from a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical except for possible naturally occurring mutations that may be present in minor amounts. Monoclonal antibodies are highly specific, being directed against a single antigenic site, i.e., the CD40 B-cell surface antigen in the present invention. Furthermore, in contrast to conventional (polyclonal) antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen. The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. For example, the monoclonal antibodies to be used in accordance with the present invention may be made by the hybridoma method first described by Kohler *et al.* (1975) *Nature* 256:495, or may be made by recombinant DNA methods (see, e.g., U.S. Patent No. 4,816,567). The "monoclonal antibodies" may also be isolated from phage antibody libraries using the techniques described in, for example, Clackson *et al.* (1991) *Nature* 352:624-628; Marks *et al.* (1991) *J. Mol. Biol.* 222:581-597; and U.S. Patent No. 5,514,548.

Monoclonal antibodies can be prepared using the method of Kohler *et al.* (1975) *Nature* 256:495-496, or a modification thereof. Typically, a mouse is immunized with a solution containing an antigen. Immunization can be performed by mixing or emulsifying the antigen-containing solution in saline, preferably in an adjuvant such as Freund's complete adjuvant, and injecting the mixture or emulsion parenterally. Any method of immunization known in the art may be used to obtain the monoclonal antibodies of the invention. After immunization of the animal, the spleen (and optionally, several large lymph nodes) are removed and dissociated into single cells. The spleen cells may be screened by applying a cell suspension to a plate or well coated with the antigen of interest. The B cells expressing membrane bound immunoglobulin specific for the antigen bind to the plate and are not rinsed away. Resulting B cells, or all dissociated spleen cells, are then induced to fuse with myeloma cells to form hybridomas, and are cultured in a selective medium. The resulting cells are plated by serial dilution and are assayed for the production of

antibodies that specifically bind the antigen of interest (and that do not bind to unrelated antigens). The selected monoclonal antibody (mAb)-secreting hybridomas are then cultured either *in vitro* (e.g., in tissue culture bottles or hollow fiber reactors), or *in vivo* (as ascites in mice).

5 As an alternative to the use of hybridomas, antibody can be produced in a cell line such as a CHO cell line, as disclosed in U.S. Patent Nos. 5,545,403; 5,545,405; and 5,998,144; incorporated herein by reference. Briefly the cell line is transfected with vectors capable of expressing a light chain and a heavy chain, respectively. By transfecting the two proteins on separate vectors, chimeric antibodies can be
10 produced. Another advantage is the correct glycosylation of the antibody.

Monoclonal antibodies to CD40 are known in the art. See, for example, the sections dedicated to B-cell antigen in McMichael, ed. (1987; 1989) *Leukocyte Typing III and IV* (Oxford University Press, New York); U.S. Patent Nos. 5,674,492; 5,874,082; 5,677,165; 6,056,959; WO 00/63395; copending U.S. Provisional Patent
15 Application Serial No. 60/237,556 entitled, "Human Anti-CD40 Antibodies," filed October 2, 2000; Gordon *et al.* (1988) *J. Immunol.* 140:1425; Valle *et al.* (1989) *Eur. J. Immunol.* 19:1463; Clark *et al.* (1986) *PNAS* 83:4494; Paulie *et al.* (1989) *J. Immunol.* 142:590; Gordon *et al.* (1987) *Eur. J. Immunol.* 17:1535; Jabara *et al.* (1990) *J. Exp. Med.* 172:1861; Zhang *et al.* (1991) *J. Immunol.* 146:1836; Gascan *et al.* (1991) *J. Immunol.* 147:8; Banchereau *et al.* (1991) *Clin. Immunol. Spectrum* 3:8; and Banchereau *et al.* (1991) *Science* 251:70; all of which are herein incorporated by reference. Any anti-CD40 antibody or antigen-binding fragment thereof having the binding characteristics and specificity noted herein is suitable for use in the methods of the present invention.

25 Additionally, the term "anti-CD40 antibody" as used herein encompasses chimeric anti-CD40 antibodies. By "chimeric" antibodies is intended antibodies that are most preferably derived using recombinant deoxyribonucleic acid techniques and which comprise both human (including immunologically "related" species, e.g., chimpanzee) and non-human components. Thus, the constant region of the chimeric
30 antibody is most preferably substantially identical to the constant region of a natural human antibody; the variable region of the chimeric antibody is most preferably derived from a non-human source and has the desired antigenic specificity to the CD40 cell-surface antigen. The non-human source can be any vertebrate source that

can be used to generate antibodies to a human CD40 cell-surface antigen or material comprising a human CD40 cell-surface antigen. Such non-human sources include, but are not limited to, rodents (e.g., rabbit, rat, mouse, etc.; see, for example, U.S. Patent No. 4,816,567, herein incorporated by reference) and non-human primates (e.g., Old World Monkey, Ape, etc.; see, for example, U.S. Patent Nos. 5,750,105 and 5,756,096; herein incorporated by reference). As used herein, the phrase "immunologically active" when used in reference to chimeric anti-CD40 antibodies means a chimeric antibody that binds human CD40.

Humanized anti-CD40 antibodies are also encompassed by the term anti-CD40 antibody as used herein. By "humanized" is intended forms of anti-CD40 antibodies that contain minimal sequence derived from non-human immunoglobulin sequences. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from a hypervariable region (also known as complementarity determining region or CDR) of the recipient are replaced by residues from a hypervariable region of a non-human species (donor antibody) such as mouse, rat, rabbit, or nonhuman primate having the desired specificity, affinity, and capacity. Humanization can be essentially performed following the method of Winter and co-workers (Jones *et al.* (1986) *Nature* 321:522-525; Riechmann *et al.* (1988) *Nature* 332:323-327; Verhoeven *et al.* (1988) *Science* 239:1534-1536), by substituting rodent or mutant rodent CDRs or CDR sequences for the corresponding sequences of a human antibody. See also U.S. Patent Nos. 5,225,539; 5,585,089; 5,693,761; 5,693,762; 5,859,205; herein incorporated by reference. In some instances, residues within the framework regions of one or more variable regions of the human immunoglobulin are replaced by corresponding non-human residues (see, for example, U.S. Patent Nos. 5,585,089; 5,693,761; 5,693,762; and 6,180,370). Furthermore, humanized antibodies may comprise residues that are not found in the recipient antibody or in the donor antibody. These modifications are made to further refine antibody performance (e.g., to obtain desired affinity). In general, the humanized antibody will comprise substantially all of at least one, and typically two, variable domains, in which all or substantially all of the hypervariable regions correspond to those of a non-human immunoglobulin and all or substantially all of the framework regions are those of a human immunoglobulin sequence. The humanized antibody optionally also will comprise at least a portion of an immunoglobulin

constant region (Fc), typically that of a human immunoglobulin. For further details see Jones *et al.* (1986) *Nature* 331:522-525; Riechmann *et al.* (1988) *Nature* 332:323-329; and Presta (1992) *Curr. Op. Struct. Biol.* 2:593-596; herein incorporated by reference. Accordingly, such "humanized" antibodies may include antibodies wherein

5 substantially less than an intact human variable domain has been substituted by the corresponding sequence from a non-human species. In practice, humanized antibodies are typically human antibodies in which some CDR residues and possibly some framework residues are substituted by residues from analogous sites in rodent antibodies. See, for example, U.S. Patent Nos. 5,225,539; 5,585,089; 5,693,761;

10 5,693,762; 5,859,205. See also U.S. Patent No. 6,180,370, and International Publication No. WO 01/27160, where humanized antibodies and techniques for producing humanized antibodies having improved affinity for a predetermined antigen are disclosed.

Also encompassed by the term anti-CD40 antibodies are xenogeneic or

15 modified anti-CD40 antibodies produced in a non-human mammalian host, more particularly a transgenic mouse, characterized by inactivated endogenous immunoglobulin (Ig) loci. In such transgenic animals, competent endogenous genes for the expression of light and heavy subunits of host immunoglobulins are rendered non-functional and substituted with the analogous human immunoglobulin loci.

20 These transgenic animals produce human antibodies in the substantial absence of light or heavy host immunoglobulin subunits. See, for example, U.S. Patent Nos. 5,877,397 and 5,939,598, herein incorporated by reference.

Fragments of the anti-CD40 antibodies are suitable for use in the methods of the invention so long as they retain the desired affinity of the full-length antibody.

25 Thus, a fragment of an anti-CD40 antibody will retain the ability to bind to the CD40 B-cell surface antigen. Such fragments are characterized by properties similar to the corresponding full-length antagonist anti-CD40 antibody, that is the fragments will 1) specifically bind a human CD40 antigen expressed on the surface of a human cell; 2) are free of significant agonist activity when bound to a CD40 antigen on a normal

30 human B cell; and 3) exhibit antagonist activity when bound to a CD40 antigen on a malignant human B cell. Where the full-length antagonist anti-CD40 antibody exhibits antagonist activity when bound to the CD40 antigen on the surface of a

normal human B cell, the fragment will also exhibit such antagonist activity. Such fragments are referred to herein as "antigen-binding" fragments.

Suitable antigen-binding fragments of an antibody comprise a portion of a full-length antibody, generally the antigen-binding or variable region thereof.

- 5 Examples of antibody fragments include, but are not limited to, Fab, F(ab')₂, and Fv fragments and single-chain antibody molecules. By "single-chain Fv" or "sFv" antibody fragments is intended fragments comprising the V_H and V_L domains of an antibody, wherein these domains are present in a single polypeptide chain. See, for example, U.S. Patent Nos. 4,946,778; 5,260,203; 5,455,030; 5,856,456; herein
- 10 incorporated by reference. Generally, the Fv polypeptide further comprises a polypeptide linker between the V_H and V_L domains that enables the sFv to form the desired structure for antigen binding. For a review of sFv see Pluckthun (1994) in *The Pharmacology of Monoclonal Antibodies*, Vol. 113, ed. Rosenberg and Moore (Springer-Verlag, New York), pp. 269-315.
- 15 Antibodies or antibody fragments can be isolated from antibody phage libraries generated using the techniques described in, for example, McCafferty *et al.* (1990) *Nature* 348:552-554 (1990) and U.S. Patent No. 5,514,548. Clackson *et al.* (1991) *Nature* 352:624-628 and Marks *et al.* (1991) *J. Mol. Biol.* 222:581-597 describe the isolation of murine and human antibodies, respectively, using phage
- 20 libraries. Subsequent publications describe the production of high affinity (nM range) human antibodies by chain shuffling (Marks *et al.* (1992) *Bio/Technology* 10:779-783), as well as combinatorial infection and *in vivo* recombination as a strategy for constructing very large phage libraries (Waterhouse *et al.* (1993) *Nucleic. Acids Res.* 21:2265-2266). Thus, these techniques are viable alternatives to traditional
- 25 monoclonal antibody hybridoma techniques for isolation of monoclonal antibodies.
- Various techniques have been developed for the production of antibody fragments. Traditionally, these fragments were derived *via* proteolytic digestion of intact antibodies (see, e.g., Morimoto *et al.* (1992) *Journal of Biochemical and Biophysical Methods* 24:107-117 (1992) and Brennan *et al.* (1985) *Science* 229:81).
- 30 However, these fragments can now be produced directly by recombinant host cells. For example, the antibody fragments can be isolated from the antibody phage libraries discussed above. Alternatively, Fab'-SH fragments can be directly recovered from *E. coli* and chemically coupled to form F(ab')₂ fragments (Carter *et al.* (1992)

Bio/Technology 10:163-167). According to another approach, F(ab')₂ fragments can be isolated directly from recombinant host cell culture. Other techniques for the production of antibody fragments will be apparent to the skilled practitioner.

Antagonist anti-CD40 antibodies useful in the methods of the present invention include the 15B8 monoclonal antibody disclosed herein as well as antibodies differing from this antibody but retaining the CDRs; and antibodies with one or more amino acid addition(s), deletion(s), or substitution(s), wherein the antagonist activity is measured by inhibition of malignant B cell proliferation and/or differentiation. The invention also encompasses de-immunized antagonist anti-CD40 antibodies, which can be produced as described in, for example, International Publication Nos. WO 98/52976 and WO 0034317; herein incorporated by reference. In this manner, residues within the antagonist anti-CD40 antibodies of the invention are modified so as to render the antibodies non- or less immunogenic to humans while retaining their antagonist activity toward malignant human B cells, wherein such activity is measured by assays noted elsewhere herein. Also included within the scope of the claims are fusion proteins comprising an antagonist anti-CD40 antibody of the invention, or a fragment thereof, which fusion proteins can be synthesized or expressed from corresponding polynucleotide vectors, as is known in the art. Such fusion proteins are described with reference to conjugation of antibodies as noted below.

Antagonist anti-CD40 antibodies identified as having the binding characteristics and specificity described herein for use in the methods of the present invention can have sequence variations produced using methods described in, for example, Patent Publication Nos. EP 0 983 303 A1, WO 00/34317, and WO 98/52976, incorporated herein by reference. For example, it has been shown that sequences within the CDR can cause an antibody to bind to MHC Class II and trigger an unwanted helper T cell response. A conservative substitution can allow the antibody to retain binding activity yet lose its ability to trigger an unwanted T cell response. Any such conservative or non-conservative substitutions can be made using art-recognized methods, such as those noted elsewhere herein, and the resulting antibodies will fall within the scope of the invention. The variant antibodies can be routinely tested for antagonist activity, affinity, and specificity using methods described herein.

An antibody produced by any of the methods described above, or any other method not disclosed herein, will fall within the scope of the invention if it possesses at least one of the following biological activities: inhibition of immunoglobulin secretion by normal human peripheral B cells stimulated by T cells; inhibition of proliferation of normal human peripheral B cells stimulated by Jurkat T cells; inhibition of proliferation of normal human peripheral B cells stimulated by CD40L-expressing cells; and inhibition of proliferation of human malignant B cells as noted below. These assays can be performed as described in the Examples herein. See also the assays described in Schultze *et al.* (1998) *Proc. Natl. Acad. Sci. USA* 92:8200-8204; Denton *et al.* (1998) *Pediatr. Transplant.* 2:6-15; Evans *et al.* (2000) *J. Immunol.* 164:688-697; Noelle (1998) *Agents Actions Suppl.* 49:17-22; Lederman *et al.* (1996) *Curr. Opin. Hematol.* 3:77-86; Coligan *et al.* (1991) *Current Protocols in Immunology* 13:12; Kwekkeboom *et al.* (1993) *Immunology* 79:439-444; and U.S. Patent Nos. 5,674,492 and 5,847,082; herein incorporated by reference.

Any of the previously described antagonist anti-CD40 antibodies or antibody fragments thereof may be conjugated prior to use in the methods of the present invention. Methods for producing conjugated antibodies are known in the art. Thus, the anti-CD40 antibody may be labeled using an indirect labeling or indirect labeling approach. By "indirect labeling" or "indirect labeling approach" is intended that a chelating agent is covalently attached to an antibody and at least one radionuclide is inserted into the chelating agent. See, for example, the chelating agents and radionuclides described in Srivagtava and Mease (1991) *Nucl. Med. Bio.* 18:589-603, herein incorporated by reference. Alternatively, the anti-CD40 antibody may be labeled using "direct labeling" or a "direct labeling approach", where a radionuclide is covalently attached directly to an antibody (typically via an amino acid residue). Preferred radionuclides are provided in Srivagtava and Mease (1991) *supra*. The indirect labeling approach is particularly preferred. See also, for example, International Publication Nos. WO 00/52031 and WO 00/52473, where a linker is used to attach a radioactive label to antibodies; and the labeled forms of anti-CD40 antibodies described in U.S. Patent No. 6,015,542; herein incorporated by reference.

Further, an antibody (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent, or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples

include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine). The conjugates of the invention can be used for modifying a given biological response; the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a protein or polypeptide possessing a desired biological activity. Such proteins may include, for example, a toxin such as abrin, ricin A, pseudomonas exotoxin, or diphtheria toxin; a protein such as tumor necrosis factor, interferon-alpha, interferon-beta, nerve growth factor, platelet derived growth factor, tissue plasminogen activator; or, biological response modifiers such as, for example, lymphokines, interleukin-1 ("IL-1"), interleukin-2 ("IL-2"), interleukin-6 ("IL-6"), granulocyte macrophage colony stimulating factor ("GM-CSF"), granulocyte colony stimulating factor ("G-CSF"), or other growth factors.

Techniques for conjugating such therapeutic moiety to antibodies are well known. See, for example, Arnon *et al.* (1985) "Monoclonal Antibodies for Immunotargeting of Drugs in Cancer Therapy," in *Monoclonal Antibodies and Cancer Therapy*, ed. Reisfeld *et al.* (Alan R. Liss, Inc.), pp. 243-256; ed. Hellstrom *et al.* (1987) "Antibodies for Drug Delivery," in *Controlled Drug Delivery*, ed. Robinson *et al.* (2d ed; Marcel Dekker, Inc.), pp. 623-653; Thorpe (1985) "Antibody Carriers of Cytotoxic Agents in Cancer Therapy: A Review," in *Monoclonal Antibodies '84: Biological and Clinical Applications*, ed. Pinchera *et al.* pp. 475-506 (Editrice Kurtis, Milano, Italy, 1985); "Analysis, Results, and Future Prospective of the Therapeutic Use of Radiolabeled Antibody in Cancer Therapy," in *Monoclonal Antibodies for*

Cancer Detection and Therapy, ed. Baldwin *et al.* (Academic Press, New York, 1985), pp. 303-316; and Thorpe *et al.* (1982) *Immunol. Rev.* 62:119-158.

Alternatively, an antibody can be conjugated to a second antibody to form an antibody heteroconjugate as described by Segal in U.S. Patent No. 4,676,980. In addition, linkers may be used between the labels and the antibodies of the invention (see U.S. Patent No. 4,831,175). Antibodies or, antigen-binding fragments thereof may be directly labeled with radioactive iodine, indium, yttrium, or other radioactive particle known in the art (U.S. Patent No. 5,595,721). Treatment may consist of a combination of treatment with conjugated and nonconjugated antibodies administered simultaneously or subsequently (WO 00/52031 and WO 00/52473).

Methods of the invention are directed to the use of antagonist anti-CD40 antibodies to treat patients having a disease comprising malignant B cells. By "malignant" B cell is intended any neoplastic B cell, including but not limited to B cells derived from lymphomas including low-, intermediate-, and high-grade B-cell lymphomas, immunoblastic lymphomas, non-Hodgkin's lymphomas, Hodgkin's disease, Epstein-Barr Virus (EBV) induced lymphomas, and AIDS-related lymphomas, as well as B-cell acute lymphoblastic leukemias, myelomas, chronic lymphocytic leukemias, acute myeloblastic leukemias, and the like.

The methods of the invention find use in the treatment of non-Hodgkin's lymphomas related to abnormal, uncontrollable B cell proliferation or accumulation. For purposes of the present invention, such lymphomas will be referred to according to the *Working Formulation* classification scheme, that is those B-cell lymphomas categorized as low grade, intermediate grade, and high grade (see "The Non-Hodgkin's Lymphoma Pathologic Classification Project," *Cancer* 49(1982):2112-2135). Thus, low-grade B-cell lymphomas include small lymphocytic, follicular small-cleaved cell, and follicular mixed small-cleaved and large cell lymphomas; intermediate-grade lymphomas include follicular large cell, diffuse small cleaved cell, diffuse mixed small and large cell, and diffuse large cell lymphomas; and high-grade lymphomas include large cell immunoblastic, lymphoblastic, and small non-cleaved cell lymphomas of the Burkitt's and non-Burkitt's type.

It is recognized that the methods of the invention are useful in the therapeutic treatment of B-cell lymphomas that are classified according to the Revised European and American Lymphoma Classification (REAL) system. Such B-cell lymphomas

include, but are not limited to, lymphomas classified as precursor B-cell neoplasms, such as B-lymphoblastic leukemia/lymphoma; peripheral B-cell neoplasms, including B-cell chronic lymphocytic leukemia/small lymphocytic lymphoma, lymphoplasmacytoid lymphoma/immunocytoma, mantle cell lymphoma (MCL),
5 follicle center lymphoma (follicular) (including diffuse small cell, diffuse mixed small and large cell, and diffuse large cell lymphomas), marginal zone B-cell lymphoma (including extranodal, nodal, and splenic types), hairy cell leukemia, plasmacytoma/myeloma, diffuse large cell B-cell lymphoma of the subtype primary mediastinal (thymic), Burkitt's lymphoma, and Burkitt's like high grade B-cell lymphoma; acute
10 leukemias; acute lymphocytic leukemias; myeloblastic leukemias; acute myelocytic leukemias; promyelocytic leukemia; myelomonocytic leukemia; monocytic leukemia; erythroleukemia; granulocytic leukemia (chronic myelocytic leukemia); chronic lymphocytic leukemia; polycythemia vera; multiple myeloma; Waldenstrom's macroglobulinemia; heavy chain disease; and unclassifiable low-grade or high-grade
15 B-cell lymphomas.

It is recognized that the methods of the invention may be useful in preventing further tumor outgrowths arising during therapy. The methods of the invention are particularly useful in the treatment of subjects having low-grade B-cell lymphomas, particularly those subjects having relapses following standard chemotherapy. Low-
20 grade B-cell lymphomas are more indolent than the intermediate- and high-grade B-cell lymphomas and are characterized by a relapsing/remitting course. Thus, treatment of these lymphomas is improved using the methods of the invention, as relapse episodes are reduced in number and severity.

The antagonist anti-CD40 antibodies described herein may also find use in the
25 treatment of inflammatory diseases and deficiencies or disorders of the immune system including, but not limited to, systemic lupus erythematosus, psoriasis, scleroderma, CREST syndrome, inflammatory myositis, Sjogren's syndrome, mixed connective tissue disease, rheumatoid arthritis, multiple sclerosis, inflammatory bowel disease, acute respiratory distress syndrome, pulmonary inflammation, idiopathic
30 pulmonary fibrosis, osteoporosis, delayed type hypersensitivity, asthma, primary biliary cirrhosis, and idiopathic thrombocytopenic purpura.

In accordance with the methods of the present invention, at least one antagonist anti-CD40 antibody (or antigen-binding fragment thereof) as defined

elsewhere herein is used to promote a positive therapeutic response with respect to a malignant human B cell. By "positive therapeutic response" is intended an improvement in the disease in association with the anti-tumor activity of these antibodies or fragments thereof, and/or an improvement in the symptoms associated with the disease. That is, an anti-proliferative effect, the prevention of further tumor outgrowths, and/or a decrease in B symptoms can be observed. Thus, for example, an improvement in the disease may be characterized as a complete response. By "complete response" is intended an absence of clinically detectable disease with normalization of any previously abnormal radiographic studies, bone marrow, and cerebrospinal fluid (CSF). Such a response must persist for at least one month following treatment according to the methods of the invention. Alternatively, an improvement in the disease may be categorized as being a partial response. By "partial response" is intended at least about a 50% decrease in all measurable tumor burden (i.e., the number of tumor cells present in the subject) in the absence of new lesions and persisting for at least one month. Such a response is applicable to measurable tumors only. In addition to these positive therapeutic responses, the subject undergoing therapy with the antagonist anti-CD40 antibody or antigen-binding fragment thereof may experience the beneficial effect of an improvement in the symptoms associated with the disease. Thus the subject may experience a decrease in the so-called B symptoms, i.e., night sweats, fever, weight loss, and/or urticaria.

By "therapeutically effective dose or amount" is intended an amount of antagonist anti-CD40 antibody or antigen-binding fragment thereof that, when administered brings about a positive therapeutic response with respect to treatment of a patient with a disease comprising malignant B cells. Administration of the pharmaceutical composition comprising the therapeutically effective dose or amount can be achieved using any acceptable administration method known in the art. Preferably the pharmaceutical composition comprising the antagonist anti-CD40 antibody or antigen-binding fragment thereof is administered intravenously, preferably by infusion over a period of about 1 to about 10 hours, more preferably over about 1 to about 8 hours, even more preferably over about 2 to about 7 hours, still more preferably over about 4 to about 6 hours, depending upon the anti-CD40 antibody being administered. The initial infusion with the pharmaceutical

composition may be given over a period of about 4 to about 6 hours with subsequent infusions delivered more quickly. Subsequent infusions may be administered over a period of about 1 to about 6 hours, preferably about 1 to about 4 hours, more preferably about 1 to about 3 hours, yet more preferably about 1 to about 2 hours.

5 A pharmaceutical composition of the invention is formulated to be compatible with its intended route of administration. Examples of routes of administration include parenteral, e.g., intravenous, intradermal, subcutaneous, oral (e.g., inhalation), transdermal (topical), transmucosal, and rectal administration. Solutions or
10 suspensions used for parenteral, intradermal, or subcutaneous application can include the following components: a sterile diluent such as water for injection, saline solution, fixed oils, polyethylene glycols, glycerin, propylene glycol or other synthetic
15 solvents; antibacterial agents such as benzyl alcohol or methyl parabens; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates or phosphates and
15 agents for the adjustment of tonicity such as sodium chloride or dextrose. pH can be adjusted with acids or bases, such as hydrochloric acid or sodium hydroxide. The parenteral preparation can be enclosed in ampoules, disposable syringes, or multiple dose vials made of glass or plastic.

 The anti-CD40 antibodies are typically provided by standard technique within
20 a pharmaceutically acceptable buffer, for example, sterile saline, sterile buffered water, propylene glycol, combinations of the foregoing, etc. Methods for preparing parenterally administrable agents are described in *Remington's Pharmaceutical Sciences* (18th ed.; Mack Publishing Company, Eaton, Pennsylvania, 1990), herein incorporated by reference. See also, for example, WO 98/56418, which describes
25 stabilized antibody pharmaceutical formulations suitable for use in the methods of the present invention.

 The amount of at least one anti-CD40 antibody or fragment thereof to be administered is readily determined by one of ordinary skill in the art without undue experimentation. Factors influencing the mode of administration and the respective
30 amount of at least one antagonist anti-CD40 antibody (or fragment thereof) include, but are not limited to, the particular lymphoma undergoing therapy, the severity of the disease, the history of the disease, and the age, height, weight, health, and physical condition of the individual undergoing therapy. Similarly, the amount of antagonist

anti-CD40 antibody or fragment thereof to be administered will be dependent upon the mode of administration and whether the subject will undergo a single dose or multiple doses of this anti-tumor agent. Generally, a higher dosage of anti-CD40 antibody or fragment thereof is preferred with increasing weight of the patient
5 undergoing therapy. The dose of anti-CD40 antibody or fragment thereof to be administered is in the range from about 0.003 mg/kg to about 50 mg/kg, preferably in the range of 0.01 mg/kg to about 40 mg/kg. Thus, for example, the dose can be 0.01 mg/kg, 0.03 mg/kg, 0.1 mg/kg, 0.3 mg/kg, 0.5 mg/kg, 1 mg/kg, 1.5 mg/kg, 2 mg/kg, 2.5 mg/kg, 3 mg/kg, 5 mg/kg, 7 mg/kg, 10 mg/kg, 15 mg/kg, 20 mg/kg, 25 mg/kg, 30
10 mg/kg, 35 mg/kg, 40 mg/kg, 45 mg/kg, or 50 mg/kg.

In another embodiment of the invention, the method comprises administration of multiple doses of antagonist anti-CD40 antibody or fragment thereof. The method may comprise administration of 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 15, 20, 25, 30, 35, 40, or more therapeutically effective doses of a pharmaceutical composition comprising an
15 antagonist anti-CD40 antibody or fragment thereof. The frequency and duration of administration of multiple doses of the pharmaceutical compositions comprising anti-CD40 antibody or fragment thereof can be readily determined by one of skill in the art without undue experimentation. Moreover, treatment of a subject with a therapeutically effective amount of an antibody can include a single treatment or,
20 preferably, can include a series of treatments. In a preferred example, a subject is treated with antagonist anti-CD40 antibody or antigen-binding fragment thereof in the range of between about 0.1 to 20 mg/kg body weight, once per week for between about 1 to 10 weeks, preferably between about 2 to 8 weeks, more preferably between about 3 to 7 weeks, and even more preferably for about 4, 5, or 6 weeks. Treatment
25 may occur annually to prevent relapse or upon indication of relapse. It will also be appreciated that the effective dosage of antibody or antigen-binding fragment thereof used for treatment may increase or decrease over the course of a particular treatment. Changes in dosage may result and become apparent from the results of diagnostic assays as described herein. Thus, in one embodiment, the dosing regimen includes a
30 first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1, 7, 14, and 21 of a treatment period. In another embodiment, the dosing regimen includes a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof

on days 1, 2, 3, 4, 5, 6, and 7 of a week in a treatment period. Further embodiments include a dosing regimen having a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1, 3, 5, and 7 of a week in a treatment period; a dosing regimen including a first administration of a
5 therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on days 1 and 3 of a week in a treatment period; and a preferred dosing regimen including a first administration of a therapeutically effective dose of at least one anti-CD40 antibody or fragment thereof on day 1 of a week in a treatment period. The treatment period may comprise 1 week, 2 weeks, 3 weeks, a month, 3 months, 6
10 months, or a year. Treatment periods may be subsequent or separated from each other by a day, a week, 2 weeks, a month, 3 months, 6 months, or a year.

The antagonist anti-CD40 antibodies present in the pharmaceutical compositions described herein for use in the methods of the invention may be native or obtained by recombinant techniques, and may be from any source, including
15 mammalian sources such as, e.g., mouse, rat, rabbit, primate, pig, and human. Preferably such polypeptides are derived from a human source, and more preferably are recombinant, human proteins from hybridoma cell lines.

The pharmaceutical compositions useful in the methods of the invention may comprise biologically active variants of the antagonist anti-CD40 antibodies of the
20 invention. Such variants should retain the desired biological activity of the native polypeptide such that the pharmaceutical composition comprising the variant polypeptide has the same therapeutic effect as the pharmaceutical composition comprising the native polypeptide when administered to a subject. That is, the variant anti-CD40 antibody will serve as a therapeutically active component in the
25 pharmaceutical composition in a manner similar to that observed for the native antagonist antibody, for example 15B8 as expressed by the hybridoma cell line 15B8. Methods are available in the art for determining whether a variant anti-CD40 antibody retains the desired biological activity, and hence serves as a therapeutically active component in the pharmaceutical composition. Biological activity of antibody
30 variants can be measured using assays specifically designed for measuring activity of the native antagonist antibody, including assays described in the present invention.

Suitable biologically active variants of native or naturally occurring antagonist anti-CD40 antibodies can be fragments, analogues, and derivatives of that

polypeptide. By "fragment" is intended a polypeptide consisting of only a part of the intact polypeptide sequence and structure, as noted elsewhere herein. By "analogue" is intended an analogue of either the native polypeptide or of a fragment of the native polypeptide, where the analogue comprises a native polypeptide sequence and
5 structure having one or more amino acid substitutions, insertions, or deletions. By "derivative" is intended any suitable modification of the native polypeptide of interest, of a fragment of the native polypeptide, or of their respective analogues, such as glycosylation, phosphorylation, polymer conjugation (such as with polyethylene glycol), or other addition of foreign moieties, so long as the desired biological activity
10 of the native polypeptide is retained. Methods for making polypeptide fragments, analogues, and derivatives are generally available in the art.

For example, amino acid sequence variants of an antagonist anti-CD40 antibody can be prepared by mutations in the cloned DNA sequence encoding the antibody of interest. Methods for mutagenesis and nucleotide sequence alterations are
15 well known in the art. See, for example, Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York); Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods Enzymol.* 154:367-382; Sambrook *et al.* (1989) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor, New York); U.S. Patent No. 4,873,192; and the references cited
20 therein; herein incorporated by reference. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the polypeptide of interest may be found in the model of Dayhoff *et al.* (1978) in *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with
25 another having similar properties, may be preferred. Examples of conservative substitutions include, but are not limited to, Gly \leftrightarrow Ala, Val \leftrightarrow Ile \leftrightarrow Leu, Asp \leftrightarrow Glu, Lys \leftrightarrow Arg, Asn \leftrightarrow Gln, and Phe \leftrightarrow Trp \leftrightarrow Tyr.

In constructing variants of the antagonist anti-CD40 antibody polypeptide of interest, modifications are made such that variants continue to possess the desired
30 activity, i.e., similar binding affinity and having the following characteristics: 1) are capable of specifically binding to a human CD40 antigen expressed on the surface of a human cell; 2) are free of significant agonist activity when bound to a CD40 antigen on a normal human B cell; and, 3) exhibit antagonist activity when bound to a CD40

antigen on a malignant human B cell. Obviously, any mutations made in the DNA encoding the variant polypeptide must not place the sequence out of reading frame and preferably will not create complementary regions that could produce secondary mRNA structure. See EP Patent Application Publication No. 75,444.

5 Biologically active variants of anti-CD40 antibodies will generally have at least 70%, preferably at least 80%, more preferably about 90% to 95% or more, and most preferably about 98% or more amino acid sequence identity to the amino acid sequence of the reference polypeptide molecule, which serves as the basis for comparison. A biologically active variant of a reference antagonist anti-CD40
10 antibody having the specificity and binding characteristics described herein may differ from the reference polypeptide by as few as 1-15 amino acids, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue. By "sequence identity" is intended the same amino acid residues are found within the variant polypeptide and the polypeptide molecule that serves as a reference when a specified,
15 contiguous segment of the amino acid sequence of the variant is aligned and compared to the amino acid sequence of the reference molecule. The percentage sequence identity between two amino acid sequences is calculated by determining the number of positions at which the identical amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched
20 positions by the total number of positions in the segment undergoing comparison to the reference molecule, and multiplying the result by 100 to yield the percentage of sequence identity.

 For purposes of optimal alignment of the two sequences, the contiguous segment of the amino acid sequence of the variants may have additional amino acid
25 residues or deleted amino acid residues with respect to the amino acid sequence of the reference molecule. The contiguous segment used for comparison to the reference amino acid sequence will comprise at least twenty (20) contiguous amino acid residues, and may be 30, 40, 50, 100, or more residues. Corrections for increased sequence identity associated with inclusion of gaps in the variant's amino acid
30 sequence can be made by assigning gap penalties. Methods of sequence alignment are well known in the art for both amino acid sequences and for the nucleotide sequences encoding amino acid sequences.

Thus, the determination of percent identity between any two sequences can be accomplished using a mathematical algorithm. One preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17. Such an algorithm is utilized in the

5 ALIGN program (version 2.0), which is part of the GCG sequence alignment software package. A PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used with the ALIGN program when comparing amino acid sequences. Another preferred, nonlimiting example of a mathematical algorithm for use in comparing two sequences is the algorithm of Karlin and Altschul (1990) *Proc.*

10 *Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403. BLAST nucleotide searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to a nucleotide sequence

15 encoding the polypeptide of interest. BLAST protein searches can be performed with the XBLAST program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to the polypeptide of interest. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-Blast can be used to perform

20 an iterated search that detects distant relationships between molecules. See Altschul *et al.* (1997) *supra*. When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., XBLAST and NBLAST) can be used. See <http://www.ncbi.nlm.nih.gov>. Also see the ALIGN program (Dayhoff (1978) in *Atlas of Protein Sequence and Structure* 5:Suppl. 3

25 (National Biomedical Research Foundation, Washington, D.C.) and programs in the Wisconsin Sequence Analysis Package, Version 8 (available from Genetics Computer Group, Madison, Wisconsin), for example, the GAP program, where default parameters of the programs are utilized.

When considering percentage of amino acid sequence identity, some amino

30 acid residue positions may differ as a result of conservative amino acid substitutions, which do not affect properties of protein function. In these instances, percent sequence identity may be adjusted upwards to account for the similarity in

conservatively substituted amino acids. Such adjustments are well known in the art. See, for example, Myers and Miller (1988) *Computer Applic. Biol. Sci.* 4:11-17.

The precise chemical structure of a polypeptide capable of specifically binding CD40 and retaining antagonist activity, particularly when bound to CD40 antigen on malignant B cells, depends on a number of factors. As ionizable amino and carboxyl groups are present in the molecule, a particular polypeptide may be obtained as an acidic or basic salt, or in neutral form. All such preparations that retain their biological activity when placed in suitable environmental conditions are included in the definition of antagonist anti-CD40 antibodies as used herein. Further, the primary amino acid sequence of the polypeptide may be augmented by derivatization using sugar moieties (glycosylation) or by other supplementary molecules such as lipids, phosphate, acetyl groups and the like. It may also be augmented by conjugation with saccharides. Certain aspects of such augmentation are accomplished through post-translational processing systems of the producing host; other such modifications may be introduced *in vitro*. In any event, such modifications are included in the definition of an anti-CD40 antibody used herein so long as the antagonist properties of the anti-CD40 antibody are not destroyed. It is expected that such modifications may quantitatively or qualitatively affect the activity, either by enhancing or diminishing the activity of the polypeptide, in the various assays. Further, individual amino acid residues in the chain may be modified by oxidation, reduction, or other derivatization, and the polypeptide may be cleaved to obtain fragments that retain activity. Such alterations that do not destroy antagonist activity do not remove the polypeptide sequence from the definition of anti-CD40 antibodies of interest as used herein.

The art provides substantial guidance regarding the preparation and use of polypeptide variants. In preparing the anti-CD40 antibody variants, one of skill in the art can readily determine which modifications to the native protein nucleotide or amino acid sequence will result in a variant that is suitable for use as a therapeutically active component of a pharmaceutical composition used in the methods of the present invention.

Any pharmaceutical composition comprising an antagonist anti-CD40 antibody as the therapeutically active component can be used in the methods of the invention. Thus liquid, lyophilized, or spray-dried compositions comprising antagonist anti-CD40 antibodies or variants thereof that are known in the art may be

prepared as an aqueous or nonaqueous solution or suspension for subsequent administration to a subject in accordance with the methods of the invention. Each of these compositions will comprise anti-CD40 antibodies or variants thereof as a therapeutically or prophylactically active component. By "therapeutically or prophylactically active component" is intended the anti-CD40 antibody or variant thereof is specifically incorporated into the composition to bring about a desired therapeutic or prophylactic response with regard to treatment, prevention, or diagnosis of a disease or condition within a subject when the pharmaceutical composition is administered to that subject. Preferably the pharmaceutical compositions comprise appropriate stabilizing agents, bulking agents, or both to minimize problems associated with loss of protein stability and biological activity during preparation and storage.

Formulants may be added to pharmaceutical compositions comprising an anti-CD40 antibody of the invention. These formulants may include, but are not limited to, oils, polymers, vitamins, carbohydrates, amine acids, salts, buffers, albumin, surfactants, or bulking agents. Preferably carbohydrates include sugar or sugar alcohols such as mono-, di-, or polysaccharides, or water soluble glucans. The saccharides or glucans can include fructose, glucose, mannose, sorbose, xylose, maltose, sucrose, dextran, pullulan, dextrin, α and β cyclodextrin, soluble starch, hydroxyethyl starch, and carboxymethylcellulose, or mixtures thereof. "Sugar alcohol" is defined as a C₄ to C₈ hydrocarbon having a hydroxyl group and includes galactitol, inositol, mannitol, xylitol, sorbitol, glycerol, and arabitol. These sugars or sugar alcohols may be used individually or in combination. The sugar or sugar alcohol concentration is between 1.0% and 7% w/v., more preferably between 2.0% and 6.0% w/v. Preferably amino acids include levorotary (L) forms of carnitine, arginine, and betaine; however, other amino acids may be added. Preferred polymers include polyvinylpyrrolidone (PVP) with an average molecular weight between 2,000 and 3,000, or polyethylene glycol (PEG) with an average molecular weight between 3,000 and 5,000. Surfactants that can be added to the formulation are shown in EP Nos. 270,799 and 268,110.

Additionally, antibodies can be chemically modified by covalent conjugation to a polymer to increase their circulating half-life, for example. Preferred polymers, and methods to attach them to peptides, are shown in U.S. Patent Nos. 4,766,106;

4,179,337; 4,495,285; and 4,609,546; which are all hereby incorporated by reference in their entireties. Preferred polymers are polyoxyethylated polyols and polyethylene glycol (PEG). PEG is soluble in water at room temperature and has the general formula: $R(O-CH_2-CH_2)_nO-R$ where R can be hydrogen, or a protective group such as an alkyl or alkanol group. Preferably, the protective group has between 1 and 8 carbons, more preferably it is methyl. The symbol n is a positive integer, preferably between 1 and 1,000, more preferably between 2 and 500. The PEG has a preferred average molecular weight between 1,000 and 40,000, more preferably between 2,000 and 20,000, most preferably between 3,000 and 12,000. Preferably, PEG has at least one hydroxy group, more preferably it is a terminal hydroxy group. It is this hydroxy group which is preferably activated to react with a free amino group on the inhibitor. However, it will be understood that the type and amount of the reactive groups may be varied to achieve a covalently conjugated PEG/antibody of the present invention.

Water-soluble polyoxyethylated polyols are also useful in the present invention. They include polyoxyethylated sorbitol, polyoxyethylated glucose, polyoxyethylated glycerol (POG), and the like. POG is preferred. One reason is because the glycerol backbone of polyoxyethylated glycerol is the same backbone occurring naturally in, for example, animals and humans in mono-, di-, triglycerides. Therefore, this branching would not necessarily be seen as a foreign agent in the body. The POG has a preferred molecular weight in the same range as PEG. The structure for POG is shown in Knauf et al. (1988) *J. Bio. Chem.* 263:15064-15070, and a discussion of POG/IL-2 conjugates is found in U.S. Patent No. 4,766,106, both of which are hereby incorporated by reference in their entireties.

Another drug delivery system for increasing circulatory half-life is the liposome. Methods of preparing liposome delivery systems are discussed in Gabizon et al. (1982) *Cancer Research* 42:4734; Cafiso (1981) *Biochem Biophys Acta* 649:129; and Szoka (1980) *Ann. Rev. Biophys. Eng.* 9:467. Other drug delivery systems are known in the art and are described in, e.g., Poznansky et al. (1980) *Drug Delivery Systems*, ed. Juliano (Oxford, New York), pp. 253-315; Poznansky (1984) *Pharm Revs* 36:277.

A further embodiment of the invention is the use of antagonist anti-CD40 antibodies for diagnostic monitoring of protein levels in tissue as part of a clinical testing procedure, e.g., to determine the efficacy of a given treatment regimen.

Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials. Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin, and aequorin; and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , or ^3H .

The antagonist anti-CD40 antibodies can be used in combination with known chemotherapeutics and cytokines for the treatment of disease states comprising malignant B cells. For example, the anti-CD40 antibodies of the invention can be used in combination with cytokines such as interleukin-2. In another embodiment, the anti-CD40 antibodies of the invention can be used in combination with Rituximab (IDEC-C2B8; IDEC Pharmaceuticals Corp., San Diego, California) or Rituxan. Rituximab is a chimeric anti-CD20 monoclonal antibody containing human IgG1 and kappa constant regions with murine variable regions isolated from a murine anti-CD20 monoclonal antibody, IDEC-2B8 (Reff *et al.* (1994) *Blood* 83:435-445).

The anti-CD40 antibodies described herein can further be used to provide reagents, e.g., labeled or labelable antibodies that can be used, for example, to identify cells expressing CD40. This can be very useful in determining the cell type of an unknown sample. Panels of monoclonal antibodies can be used to identify tissue by species and/or by organ type. In a similar fashion, these anti-CD40 antibodies can be used to screen tissue culture cells for contamination (i.e., screen for the presence of a mixture of CD40-expressing and non-CD40 expressing cells in a culture).

The following examples are offered by way of illustration and not by way of limitation.

EXPERIMENTAL

Example 1: Effect of Antagonist Anti-CD40 Antibody on the CD40/CD40L

5

Interaction *In Vitro*

A competitive binding assay is performed to determine if direct competition for CD40 binding is a mechanism of the antagonist activity of a previously identified antagonist anti-CD40 antibody.

A line of Chinese Hamster Ovary (CHO) cells containing the gene encoding CD40L and expressing CD40L on the cell surface is generated. The CD40L-expressing CHO cells are incubated with purified CD40 before and after incubation of CD40 with the anti-CD40 antibody. Fluorescein isothiocyanate (FITC)-labeled anti-huIgG is added to the cells. FACS analysis is performed to detect anti-CD40 antibody bound to the CHO cells via CD40. The binding of the anti-CD40 antibody to CD40 inhibits the subsequent binding of CD40L to CD40. However, when CD40L and CD40 are incubated together prior to the addition of anti-CD40 antibody, anti-CD40 antibody is subsequently able to bind CD40. While not bound by any mechanism of action, this suggests that in this case the anti-CD40 antibody does not compete directly with CD40L for binding sites on CD40, and that the binding of this anti-CD40 antibody to CD40 possibly causes conformational changes in the CD40 molecule that prevent the binding of CD40L to CD40. The putative structural alteration of the CD40 molecule induced by anti-CD40 antibody binding could also deliver a negative signal to the cell causing the antagonist effect.

25 Example 2: Effects of Anti-CD40 Antibody on B-Cell Proliferation

Human B cells are obtained from the peripheral blood of healthy volunteers. Freshly isolated B cells are cultured at a density of 4×10^4 cells/well in IMDM media supplemented with 10% fetal calf serum in 96-well microtiter plates. The B cells are stimulated by addition of immobilized anti-IgM antibodies (Immunobeads obtained from BioRad, Inc.). Varying concentrations of antagonist anti-CD40 antibody, CD40L, or an agonist anti-CD40 antibody are added at the onset of the microcultures. Proliferation is assessed at day 3 by measurement of the incorporation of ^3H methyl-thymidine after an 18 hour pulse. The antagonist anti-CD40 antibody inhibits

proliferation of these B cells, while B-cell proliferation is increased in the presence of CD40L or the agonist anti-CD40 antibody.

Example 3: Assessment of B-cell Activation Markers upon Incubation
with an Anti-CD40 Antibody

When B-cells are activated, a number of cell surface proteins that are B-cell activation markers are up-regulated. Antagonist anti-CD40 antibody is tested for up-regulation of the B-cell activation markers CD25, CD69, CD86, HLA-DR, and ICAM-1 on purified human peripheral blood mononuclear cells. The expression of the activation markers on purified human peripheral blood mononuclear cells after incubation with the anti-CD40 antibody is determined by FACS analysis. There is no difference in the expression level of these markers between antagonist anti-CD40 antibody-treated and isotype control antibody-treated cells.

Example 4: Human T-Cell Helper-Mediated Antibody Production in Cells

When B cells are activated, they undergo a number of biological responses such as proliferation and antibody production. The activation of B cells by T-dependent antigens involves CD4⁺ T-helper (Th) cells. This T-cell help process is mediated partly by the interaction of CD40 on the B cells with the CD40L on the Th cells.

Purified human peripheral blood B cells are cultured in the presence of purified irradiated T cells activated with anti-CD3 antibody. After 8 days, cells are spun down, and the cell-free supernatant is harvested. The level of IgM production is assessed by ELISA. Antagonist anti-CD40 antibody reduces IgM production by about 30%. Therefore, this type of antibody can reduce T cell-mediated B-cell immunoglobulin production.

Example 5: Demonstration of Antagonist Activity of an Anti-CD40
Antibody in Human B Cells *In Vitro*

To demonstrate antagonist activity of an anti-CD40 antibody of interest, the ability of the antibody to inhibit CD40-CD40L interaction is tested in a CD40L-mediated human B-cell proliferation assay (Kwekkeboom *et al.* (1993) *Immunology* 79:439-444). A transfected CHO cell line expressing human CD40L is used to

stimulate the proliferation of purified human peripheral blood B cells or PBMCs. Human B cells from 10 healthy volunteers and human PBMCs from 10 healthy volunteers are tested. In all the samples tested, the anti-CD40 antibody of interest suppresses CD40L-expressing CHO cells mediated-proliferation by at least 30% at concentrations ranging from 0.2 – 5 $\mu\text{g/ml}$. This observation demonstrates that an antagonist anti-CD40 antibody can inhibit the growth signals in human B cells and PBMCs provided by cell surface-expressed CD40L.

Example 6: Effect of Antagonist Anti-CD40 Antibody on Human
Malignant B-Cell Proliferation *In Vitro*

Malignant B cells from tumor infiltrated lymph nodes (NHL cells) are obtained from 10 non-Hodgkin's lymphoma (NHL) patients. The NHL cells are studied under four different culture conditions: no added antibody (medium); addition of human isotype antibody IgG2 (control; referred to as huIgG2); addition of anti-CD40 antibody MS81 (agonist antibody); and addition of the antagonist anti-CD40 antibody of interest. All antibodies are tested at 1, 2, and 5 $\mu\text{g/ml}$ in the presence of human IL-4 (2 ng/ml) or absence of IL-4. B-cell proliferation is measured by ^3H -thymidine incorporation (Schultze *et al.* (1995) *Proc. Natl. Acad. Sci. USA* 92:8200-8204).

Antagonist anti-CD40 antibody, at concentrations of 1, 2, and 5 $\mu\text{g/ml}$, does not stimulate NHL cells to proliferate in either the absence or presence of IL-4. In contrast, the agonist anti-CD40 antibody (MS81), tested at the same concentration, stimulates NHL-cell proliferation both in the presence and in the absence of IL-4 in all patient samples. These results confirm that the anti-CD40 antibody of interest is not an agonist anti-CD40 antibody and does not stimulate proliferation of NHL cells from NHL patients *in vitro*.

FACS analysis of the NHL cells is performed with either a direct-labeled antagonist anti-CD40 antibody-FITC or antagonist anti-CD40 antibody plus anti-huIgG2-FITC to confirm that CD40 is expressed on the surface the NHL cells tested and that the antagonist antiCD40 antibody binds to the NHL cells. NHL cells from all the patients express CD40 and bind the antagonist anti-CD40 antibody.

Example 7: Antagonist Anti-CD40 Antibody Inhibits CD40L-Stimulated
Proliferation of Human NHL Cells *In Vitro*

Malignant B cells (NHL cells) from 10 NHL patients are cultured as described above in suspension over CD40L-expressing feeder cells under four different
5 conditions: no added antibody (medium); addition of human isotype antibody IgG2 (control); addition of anti-CD40 antibody MS81 (agonist antibody); and addition of the antagonist anti-CD40 antibody of interest. All antibodies are added at concentrations of 1, 2, and 5 $\mu\text{g/ml}$ in the presence of human IL-4 (2 ng/ml) or in the absence of human IL-4. The NHL cell proliferation is measured by ^3H -thymidine
10 incorporation as noted in Example 4 above. The antagonist anti-CD40 antibody inhibits the proliferation of NHL cells when compared to the control in a dose-dependent manner, as the inhibitory effect increases with increasing antagonist anti-CD40 antibody dose.

15 Example 8: Effect of Anti-CD40 Antibody on Peripheral B Cells in Chimpanzees

Two groups of male chimpanzees receive either 0.03 mg/kg or 3 mg/kg antagonist anti-CD40 antibody by intravenous administration. Serum anti-CD40 antibody concentrations and peripheral B cell numbers are monitored immediately after antibody administration and through day 28 post-dose. After administration of
20 anti-CD40 antibody at 3 mg/kg, serum anti-CD40 antibody concentrations decline. Peripheral B-cell numbers decrease immediately after antagonist anti-CD40 antibody administration and recover within 3-4 weeks. Anti-CD40 antibody is detected in serum, bound to surface CD40 receptors on circulating B cells. The extent of binding appears to remain relatively unchanged in the first week post-dose, then declines
25 subsequently through Day 28 post-dose.

After administration of anti-CD40 antibody at 0.03 mg/kg, B cells appear to decline slightly by 30 minutes but return to pre-dose values within 4-6 hours. Serum anti-CD40 antibody concentrations are below the level of detection at 30 minutes after dosing.

30

Example 9: ELISA Assay for Immunoglobulin Quantification

The concentrations of human IgM and IgG are estimated by ELISA assays. 96-well ELISA plates are coated with 4 $\mu\text{g/ml}$ anti-human IgG mAb or with 1.2 $\mu\text{g/ml}$

anti-human IgM mAb in 0.05 M carbonate buffer (pH=9.6) for 16 hours at 4°C.

Plates are washed three times with PBS-0.05% Tween-20 (PBS-Tween) and saturated with BSA for one hour. After two washes, the plates are incubated for one hour at 37°C with different dilutions of the test samples. After three washes, bound Ig is

- 5 detected by incubation for one hour at 37°C with 1 µg/ml peroxidase labeled mouse anti-human IgG mAb or mouse anti-human IgM mAb. Plates are washed four times and bound peroxidase activity is revealed by the addition of o-phenylenediamine as a substrate.

- 10 All publications and patent applications mentioned in the specification are indicative of the level of those skilled in the art to which this invention pertains. All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

- 15 Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

THAT WHICH IS CLAIMED:

1. A method for treating a patient with a disease comprising malignant B cells, said method comprising administration of a therapeutically effective amount
5 of an anti-CD40 antibody or antigen-binding fragment thereof to said patient, wherein said anti-CD40 antibody or fragment thereof is free of significant agonist activity when said antibody binds a CD40 antigen on a normal human B cell and wherein said anti-CD40 antibody or fragment thereof exhibits antagonist activity when said antibody binds a CD40 antigen on a malignant human B cell, wherein said method for
10 treating said patient promotes a positive therapeutic response in said patient.

2 The method of claim 1, wherein said anti-CD40 antibody or fragment thereof exhibits antagonist activity when said antibody binds said CD40 antigen on said normal human B cell.
15

3. The method of claim 1, wherein said antibody has a dissociation constant (K_d) of at least about 10^{-5} M.

4. The method of claim 3, wherein said antibody has a K_d of at least
20 about 10^{-8} M to about 10^{-20} M.

5. The method of claim 4, wherein said antibody has a K_d of at least about 5×10^{-9} M to about 10^{-16} M.

25 6. The method of claim 1, wherein said anti-CD40 antibody is a human anti-CD40 monoclonal antibody or antigen-binding fragment thereof.

7. The method of claim 1, wherein said antibody is a humanized anti-CD40 monoclonal antibody or antigen-binding fragment thereof.
30

8. The method of claim 1 wherein said malignant B cells are selected from the group comprising B-cell lymphoma cells, non-Hodgkin's lymphoma cells, high-grade B-cell lymphoma cells, intermediate-grade B-cell lymphoma cells, low-

grade B-cell lymphoma cells, B-cell acute lymphoblastic leukemia cells, multiple myeloma cells, chronic lymphocytic leukemia cells, myeloblastic leukemia cells, and Hodgkin's disease cells.

5 9. The method of 1, wherein said treatment comprises administering to said patient at least one therapeutically effective dose of a pharmaceutical composition comprising said anti-CD40 antibody or fragment thereof.

10 10. The method of 9, wherein said therapeutically effective dose of said anti-CD40 antibody or fragment thereof is in the range from about 0.01 mg/kg to about 40 mg/kg.

15 11. The method of 1, wherein said treatment comprises administration of multiple therapeutically effective doses of said anti-CD40 antibody or fragment thereof.

 12. The method of 1, wherein said anti-CD40 antibody is an immunologically active chimeric anti-CD40 antibody.

20 13. The method of claim 1, wherein said antigen-binding fragment is selected from the group consisting of a Fab fragment, an F(ab')₂ fragment, an Fv fragment, and a single-chain Fv fragment.

25 14. A method of inhibiting proliferation of malignant cells of B cell lineage, said method comprising contacting said malignant cells with an effective amount of an anti-CD40 antibody or antigen-binding fragment thereof, said antibody being free of significant agonistic activity, whereby when said antibody binds to CD40 antigen on said malignant cells, the proliferation of said malignant cells is inhibited.

30

 15. The method of claim 14, wherein said malignant cells are selected from the group consisting of B-cell lymphoma cells, non-Hodgkin's lymphoma cells, high-grade B-cell lymphoma cells, intermediate-grade B-cell lymphoma cells, low-

grade B-cell lymphoma cells, B-cell acute lymphoblastic leukemia cells, multiple myeloma cells, chronic lymphocytic leukemia cells, myeloblastic leukemia cells, and Hodgkin's disease cells.

5 16. The method of claim 14, wherein said anti-CD40 monoclonal antibody has a dissociation constant (K_d) of at least about 10^{-5} M.

 17. The method of claim 16, wherein said anti-CD40 monoclonal antibody has a K_d of at least about 10^{-8} M to about 10^{-20} M.

10

 18. The method of claim 17, wherein said anti-CD40 monoclonal antibody has a K_d of at least about 5×10^{-9} M to about 10^{-16} M.

 19. The method of claim 14, wherein said anti-CD40 antibody is a human
15 anti-CD40 monoclonal antibody or antigen-binding fragment thereof.

 20. The method of claim 14, wherein said anti-CD40 antibody is a humanized anti-CD40 monoclonal antibody or antigen-binding fragment thereof.

20 21. The method of claim 14, wherein said anti-CD40 antibody is an immunologically active chimeric anti-CD40 antibody.

THIS PAGE BLANK (USPTO)

THIS PAGE BLANK (USPTO)
THIS PAGE BLANK (USPTO)

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
11 April 2002 (11.04.2002)

PCT

(10) International Publication Number
WO 02/028480 A3

(51) International Patent Classification⁷: **A61P 35/00**,
A61K 39/395 // C07K 16/28

(21) International Application Number: **PCT/US01/30961**

(22) International Filing Date: 2 October 2001 (02.10.2001)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/237,556 2 October 2000 (02.10.2000) US

(71) Applicant (for all designated States except US): **CHIRON CORPORATION** [US/US]; 4560 Horton Street, Emeryville, CA 94608-2916 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **CHU, Keting** [US/US]; Chiron Corporation, P.O. Box 8097, Emeryville, CA 94622-8097 (US). **MASUOKA, Lorianne** [US/US]; Chiron Corporation, P.O. Box 8097, Emeryville, CA 94622-8097 (US).

(74) Agents: **ALEXANDER, Lisa, E.**; Chiron Corporation, Intellectual Property, P.O. Box 8097, Emeryville, CA 94662-8097 et al. (US).

(81) Designated States (national): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, FR, GB, GR, GU, HK, HN, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MY, NZ, NO, NZ, PH, PT, RO, RU, SD, SE, SG, SI, SK, SL, SM, ST, SV, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA.

(84) Designated States (regional): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

- with international search report
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments

(88) Date of publication of the international search report:
11 July 2002

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: **METHODS OF THERAPY FOR B-CELL MALIGNANCIES**

(57) Abstract: Methods of therapy for B-cell malignancies are provided. The methods comprise administering a therapeutically effective amount of an antagonist anti-CD40 antibody or antigen-binding fragment thereof to a patient in need thereof. The antagonist anti-CD40 antibody or antigen-binding fragment thereof is free of significant agonist activity when the antibody binds a CD40 antigen on a normal human B cell, exhibits antagonist activity when the antibody binds a CD40 antigen on a malignant human B cell, and can exhibit antagonist activity when the antibody binds a CD40 antigen on a normal human B cell. Antagonist activity of the anti-CD40 antibody or antigen-binding fragment thereof beneficially inhibits proliferation and/or differentiation of malignant human B cells.

WO 02/028480 A3

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/30961

A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 A61P35/00 A61K39/395 //C07K16/28

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

BIOSIS, EMBASE, WPI Data, PAJ, EPO-Internal

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	S. FUNAKOSHI ET AL.: "Inhibition of human B-cell lymphoma growth by CD40 stimulation." BLOOD, vol. 83, no. 10, 15 May 1994 (1994-05-15), pages 2787-2794, XP001064643 New York, NY, USA abstract materials and methods - antibodies ---	1-5, 8-11, 13-18
A	EP 0 945 465 A (CHIRON CORPORATION) 29 September 1999 (1999-09-29) examples claims --- -/--	1-5, 7-18, 20, 21

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

* Special categories of cited documents:

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- * & * document member of the same patent family

Date of the actual completion of the international search

15 April 2002

Date of mailing of the international search report

29/04/2002

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,
Fax: (+31-70) 340-3016

Authorized officer

Nooij, F

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 01/30961

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	J. KWEKKEBOOM ET AL.: "CD40 plays an essential role in the activation of human B cells by murine EL4B5 cells." IMMUNOLOGY, vol. 79, no. 3, July 1993 (1993-07), pages 439-444, XP002029781 Oxford, GB cited in the application the whole document	1-21
P,X	WO 01 24823 A (CHIRON CORPORATION) 12 April 2001 (2001-04-12) page 3, line 22 - line 24 page 7, line 18 - line 20 examples claims	1-21
E	WO 01 83755 A (GEMINI SCIENCE, INC.) 8 November 2001 (2001-11-08) example 3 claims	1,2,6,8, 9,13-15, 19

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/US 01/30961

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
EP 945465	A	29-09-1999	US 5397703 A	14-03-1995
			US 5869050 A	09-02-1999
			US 5677165 A	14-10-1997
			EP 0945465 A1	29-09-1999
			CA 2125472 A1	20-01-1994
			EP 0651797 A1	10-05-1995
			JP 7509359 T	19-10-1995
			WO 9401547 A2	20-01-1994
			US 5747034 A	05-05-1998
			US 6056959 A	02-05-2000
			US 6004552 A	21-12-1999
			US 6315998 B1	13-11-2001
			US 5874082 A	23-02-1999
WO 0124823	A	12-04-2001	AU 1072701 A	10-05-2001
			WO 0124823 A1	12-04-2001
WO 0183755	A	08-11-2001	AU 5921501 A	12-11-2001
			WO 0183755 A2	08-11-2001